

**NEW STRAINS OF SACCHARAOTHRIX, PROCESS FOR
PRODUCING PRAVASTAIN USING THE STRAINS AND
ISOLATION PROCESS OF (HMG)-COA REDUCTASE**

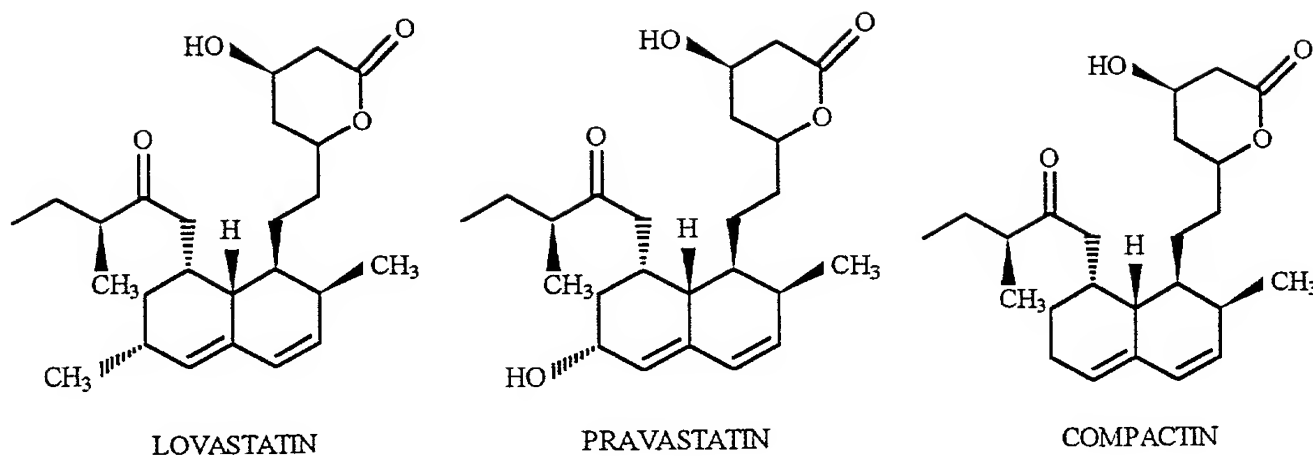
Field of the Invention

5 The present invention relates to two new microorganism strains of *Saccharothrix*, designated as *YS-44442* and *YS-45494*, a process of producing pravastatin using the strains, and an improved process for isolation of (HMG)-CoA reductase inhibitors.

Background of the invention

10 It has been recognized that an elevated blood cholesterol level is one of the major risk factors to atherosclerotic diseases, specifically to coronary heart diseases. The monitor for the cholesterol biosynthesis is very helpful to control the diseases. 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase is the rate-limiting enzyme in the cholesterol biosynthesis. By
15 inhibiting the activity of (HMG)-CoA reductase, blood cholesterol levels in the bodies can be effectively reduced.

 A number of (HMG)-CoA reductase inhibitors have been discovered, such as pravastatin, compactin, lovastatin. They have the following formula in the lactone form and may exist in other forms such as the acid
20 form or and the salts and esters thereof.



These (HMG)-CoA reductase inhibitors are very effective in lowering blood cholesterol level in most animals including human. Pravastatin is even more active than compactin and lovastatin, and has been applied in the treatment of hypercholesterolemia (Nara, F et al. *Curr. Genet.* **23**: 28-32 (1993)).

Pravastatin is a 3 β -hydroxy derivative of compactin. It has been reported that pravastatin is produced by converting compactin through microbial hydroxylation using various genera of fungi and bacteria, such as *Streptomyces roseochromogenus* (USP 4,346,277) and *Actinomadura* sp. (Peng, M. et al., *J. Antibiotics*, **Dec**: 1032-1035 (1997), and Peng Y. and A. L. Demain, *J. Mol. Catal. B: Enzymatic* **10**: 151-156 (2000)). However, these fungi or bacteria do not tolerate a high amount of compactin added in the fungal or bacterial fermentation broth, likely due to the anti-fungal activity of compactin, and thus exhibit low productivity of pravastatin. Therefore, there is a need to find a new microorganism which is tolerable to a higher amount of compactin and has effective conversion activity.

In general, the isolation of (HMG)-CoA reductase inhibitors from a fermentation broth is conducted by serious procedures of extraction, chromatography, lactonization and crystallization process. EP 0 877

089A1 discloses a (HMG)-CoA reductase inhibitor preparation process, wherein a fermentation broth containing the inhibitor (e.g., lovastatin) is basified prior to filtration to remove the cells and then the filtrate was loaded through a column. The eluate was extracted with toluene and subsequently the lactonization is conducted to produce the inhibitor.

When using chromatography, however, a large column and a great volume of fermentation broth containing (HMG)-CoA reductase inhibitors are usually needed to obtain a desired yield of the inhibitors, thereby increasing the difficulty in handling the purification process of the inhibitors. Furthermore, a lactonization reaction usually needs much energy and time. Therefore, there is a need to seek an improved process of obtaining a (HMG)-CoA reductase inhibitor in a good yield and purity without proceeding a lactonization reaction and using a chromatography, and so as to reduce the cost.

SUMMARY OF THE INVENTION

One object of the invention is to provide two new microorganism strains of *Saccharothrix* capable of producing pravastatin designated as YS-44442 and YS-45494 as well as their mutants.

Another object of the invention is to provide a process of producing pravastatin by using the microorganisms of the invention. In particular, the process of producing pravastatin comprises the steps of (a) cultivating YS-44442 or YS-45494 at a suitable condition to generate a fermentation broth; (b) feeding compactin into the broth; (c) fermenting the broth for a period of time to convert the compactin to pravastatin; (d) isolating the pravastatin from the broth.

Still another object of the invention is to provide a process of isolating a (HMG)-CoA reductase inhibitor comprising the steps of (1) adding an ammonium sulfate into a first solution containing the (HMG)-CoA reductase inhibitor to produce a precipitation; (2) isolating the precipitation; (3) dissolving the precipitation with a polar solvent to produce a second solution; (4) adjusting the pH of the second solution to about 4 to about 6; and (5) extracting the second solution with an water immiscible solvent to isolate the (HMG)-CoA reductase inhibitor. The preparation process further comprises a step of reacting the isolated (HMG)-CoA reductase inhibitor with an organic or inorganic cation source to generate a salt form of the inhibitor.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a 16s rDNA phylogenetic distance tree comparing *YS-44442* and *YS-45494* of the invention with related actinomycetes generated with the ARB software package (denoted as Tree 0.1 version).

DETAILED DESCRIPTION OF THE INVENTION

The present invention will be more fully understood and further advantages will become apparent when reference is made to the following description of the invention.

I. New microorganism strains

One object of the invention is to provide two new microorganism strains for producing pravastatin in a good yield. The inventors surprisingly obtained two novel microorganism strains, *YS-44442* and *YS-45494*, through the isolation of actinomycetes from soil samples,

showing a high tolerance to compactin and a high productivity of pravastatin. The two strains were deposited with the China Center for Type Culture Collection (CCTCC) under the accession number of M202001 and M202002, respectively, on 8 January 2002.

5 *YS-44442* and *YS-45494* can be isolated from soils. A soil sample is suspended in a sterile phosphate buffer and spread, after a 10-fold serial dilution, on an agar plate containing an isolation medium. The colonies growing on the plates can be preliminarily screened based on the morphological characteristics of actinomycetes under a microscopy
10 examination. A candidate strain is then cultivated to give a fermentation broth and fed with compactin to produce pravastatin. The amount of pravastatin thus produced in the broth is determined. Then, *YS-44442* and *YS-45494* are selected for their high productivities of pravastatin.

 The medium of the agar plate is well known in this art, preferably
15 containing yeast extract, malt extract, dextrose and agar, and more preferably ISP 2 medium. In certain embodiments, the isolation reagent contained in the medium preferably contains antibiotics which can be selected from nystatin, cycloheximide, penicillin G, polymyxin B and gentamycin. The morphological characteristics of the genus,
20 *Actinomycetes*, have been well described in conventional literatures or text books, such as Bergey's Manual of Systematic Bacteriology [Cross, T., *Bergey's Manual of Systematic Bacteriology*, S.T. Williams, Sharpe, M.E., Holt, J.G., Editor (1989) Williams & Wilkins: Baltimore. p. 2586-2615]. In addition, the technologies of cultivation or fermentation of such
25 microorganisms are well known in the art.

The compactin to be fed into a broth is preferably provided by a microorganism (e.g., fungus or bacterium) producing compactin or the cell extract thereof, or a solution comprising compactin. The concentration of compactin fed into a broth is calculated based on the broth without microbe and only the feeding compactin. Pravastatin produced in a fermentation broth can be identified by conventional technologies, such as HPLC, and the amount of pravastatin can be determined by the retention time in comparison with a standard preparation. For example, the yield of pravastatin produced can be determined by Formula (I). In addition, the conversion efficiency of compactin to pravastatin can be calculated by Formula (II) and the epi-pravastatin ratio(Epi %) of the total pravastatin produced is calculated by the formula (III).

Pravastatin yield = (standard concentration/standard Area)*sample area (I)

Molar conversion efficiency(ME%)=(Number of moles of pravastatin produced/ Number of moles of compactin converted) *100 (II)

Epi-pravastatin ratio(Epi %)=(amount of Epi-pravastatin produced/ (amount of Epi-pravastatin produced + amount of pravastatin produced))*100 % (III)

Epi-pravastatin is a 6 α -hydroxylation form of compactin which does not have a desired therapeutic efficacy and even has a negative effect in clinical therapies. Therefore, a low epi-pravastatin ratio is preferred. In addition, the term "compactin tolerance" used herein means a final concentration of compactin fed into a microbial fermentation broth wherein the concentration is accepted by the microorganism, i.e., the

microorganism can normally grow in a medium containing such amount of compactin.

5 The *YS-44442* obtained as described above has the following properties: the colonies (7 days) on plates are pale white without sporulation; the culture in SCY agar slant are pale white with few spores; the conversion efficiency of compactin to pravastatin (ME %) is about 70 to 75 %; the epi-pravastatin ratio is 6.5 to 8 %; the production of pravastatin is 1.0 to 1.5 g/L by volume of the fermentation broth; and the compactin tolerance is 1.5 to 2.0 g/L. More detailed characteristics of
10 *YS-44442* are described in the following examples.

The *YS-45494* obtained as described above has the following properties: the colonies (7 days) on plates are white with sporulation; the culture in SCY agar slant produces white spores and yellow pigments; the conversion efficiency of compactin to pravastatin (ME %) is about 48 to 50
15 %; the epi-pravastatin ratio is 1.8 to 3.0 %; the production of pravastatin is 1.0 to 1.5 g/L by volume of the fermentation broth; and the compactin tolerance reaches 1.5 to 2.0 g/L. More detailed characteristics of *YS-45494* are described in the following examples.

Mutants derived from *YS-44442* and *YS-45494* capable of producing
20 pravastatin are also included in the invention. The mutants can be obtained by applying a conventional mutation-inducing technique to the parent *YS-44442* and *YS-45494*. For example, irradiation of the microorganisms with gamma rays or ultraviolet light, or treated with a mutagen, such as EMS (ethylmethane sulfonate), NTG
25 (N-methyl-N-nitro-N'-nitrosoguanidine, NQO (4-nitroquinoline N-oxide),

DES (diethylsulfate), DEB (diepoxybutane) and NMU (N-methyl-N-nitrosourea) are suitable methods of inducing mutation. To obtain a mutant, the parent strain *YS-44442* and *YS-45494* may be treated with UV irradiation for 30, 60, 120, 240 or 480 seconds, or with cobalt 60 gamma irradiation in an amount of 0.5, 1, 2, 3 or 4 Kgy. A desirable mutant strain capable of producing pravastatin in good yield can be selected by the method as described above. Persons skilled in the art can obtain such mutants from the parent *YS-44442* and *YS-45494* according to the mutagenesis technologies and screening method conventional to the art. More detailed procedures of the mutagenesis procedures are described in the following examples.

II. Process for producing pravastatin using the strains of the invention

YS-44442 and *YS-45494* can be used to produce pravastatin. Accordingly, one object of the invention is to provide a process of producing pravastatin of using *YS-44442* and *YS-45494*. In particular, the process comprises the steps of (a) cultivating *YS-44442* or *YS-45494* in a suitable condition to generate a fermentation broth; (b) feeding compactin into the broth; (c) fermenting the broth for a period of time to convert the compactin to the pravastatin; (d) isolating the pravastatin from the broth.

The technologies of cultivating such microorganisms and those of isolating pravastatin from a fermentation broth are well known in the art. Persons skilled in the art can accomplish the production process of pravastatin using *YS-44442* and *YS-45494* of the invention in combination with any fermentation and isolation technologies (e.g., HPLC) known in the art. A particular example is illustrated later.

In one embodiment of the invention, the fermentation broth of Step (a) is incubated for less than 2 days and preferably for about 18 hours. The fermentation broth of Step (a) is preferably derived from a seed culture of the microorganism which is cultivated under a suitable condition for about 18 to 48 hrs. A seed medium for cultivating the seed culture can contain glucose, peptone, soy protein and mineral sources. A fermentation medium can further contain corn steep powder (C.S.P.).

The compactin to be added in the broth is preferably provided by a fungus or bacterium capable of producing compactin or the cell extract thereof, or a solution comprising compactin. The concentration of compactin fed in a broth is calculated based on the broth without microbe and only the feeding compactin. In a preferred embodiment of the invention, the compactin is fed into the broth in a final concentration of higher than 1.0 g/L and more preferably in a range of 1.5 to 2.0 g/L.

In another embodiment of the invention, the period of time of Step (c) to convert the compactin to the pravastatin is less than 5 days, preferably less than 3 days and more preferably less than 24 hours.

In one preferred embodiment of the invention, the pravastatin isolated from Step (d) yields more than 0.5 g/L, preferably more than 1.0 g/L and most preferably about 1.5 g/L. In one preferred embodiment of the invention, the epi-pravastatin ratio (EP %) of the total pravastatin produced from Step (d) is about 1.8 to 8 %. In one preferred embodiment of the invention, the conversion efficiency (ME %) of compactin to pravastatin is higher than 40 %, more preferably higher than 50 %, still more preferably higher than 70 %. The pravastatin produced from the

above process can be isolated by any conventional technologies in the art, such as HPLC, and further extracted or crystallized.

III. Process for isolating pravastatin without using chromatography

5 The inventors developed an improved process of isolating a (HMG)-CoA reductase inhibitor in a good yield and purity without using a chromatography and proceeding a lactonization reaction.

Accordingly, one object of the invention is to provide a process of isolating a (HMG)-CoA reductase inhibitor which comprises the steps of (1) adding an ammonium sulfate into a first solution containing
10 the (HMG)-CoA reductase inhibitor to produce a precipitation; (2) isolating the precipitation; (3) dissolving the precipitation with a polar solvent to produce a second solution; (4) adjusting the pH of the second solution to about pH 4 to about PH 6; and (5) extracting the second solution with an water immiscible solvent to isolate the (HMG)-CoA reductase inhibitor.

15 In a preferred embodiment of the invention, the (HMG)-CoA reductase inhibitors is selected from pravastatin, compactin and lovastatin, and more preferably pravastatin.

The first solution of Step (1) of the present isolation process can be any solution containing a (HMG)-CoA reductase inhibitor to be isolated.
20 In a preferred embodiment of the invention, the first solution of Step (1) of the present isolation process is a microbial fermentation broth which can be derived from any microorganism capable of producing the (HMG)-CoA reductase inhibitor. The microorganism is preferably selected from *Streptomyces roseochromogenus* (USP 4,346,277), *Actinomadura* sp.

(Peng, M. et al. *supra* and Peng Y. and A. L. Demain, *supra*). *Aspergillus*,
Monascus, *Penicillium*, *Paecilomyces*, *Hypomyces*, *Phoma*, *Pleurotus*,
Doratomyces, *Eupenicillium*, *Gymnoaxus*, *Trichoderma* (EP 0 877 089 A1),
more preferably YS-44442 and YS-45494 of the invention, and their
5 mutants described herein.

The ammonium sulfate of Step (1) is preferably added into the first
solution in an amount of 30 to 60 % (w/v) of the first solution. More
preferably, the ammonium sulfate is added to be saturated in the first
solution.

10 In Step (1) of the present isolation process, the precipitation can be
isolated by any method known in this art, such as filtration, centrifugation
or decantation, and a membrane filtration is preferred.

In a preferred embodiment of the invention, the pH of the second
solution of Step (4) is adjusted with an inorganic acid, preferably HCl.
15 Persons skilled in the art can select a proper acid according to conventional
technologies and knowledge to adjust the pH of the second solution.

In Step (5) of the present isolation process, the technology used to
extract the second solution with an water immiscible solvent is well known
in this art. Persons skilled in the art can select and use a proper water
20 immiscible solvent to successfully extract the (HMG)-CoA reductase
inhibitor from the second solution. Preferably, the water immiscible
solvent is an organic solvent which can be selected from ethyl acetate,
acetone, toluene, dichloromethane and isopropyl acetate, and more
preferably ethyl acetate. The amount of the organic solvent to be added is
25 dependent on the concentration of the (HMG)-CoA reductase inhibitor

contained in the second solution. The time period of extraction is preferably more than 5 minutes, most preferably 5 to 30 minutes.

After extraction with an organic solvent, the organic solvent layer is collected, and then dried and de-colored using the conventional technologies (such as anhydrous magnesium sulfate and activated carbon) to obtain an isolated (HMG)-CoA reductase inhibitor.

In one embodiment of the invention, the present isolation process further comprises a step of reacting the isolated (HMG)-CoA reductase inhibitor with an organic or inorganic cation source, preferably sodium, to generate a salt form of the inhibitor. The sodium source is preferably selected from NaOH, Na₂CO₃, sodium acetate (anhydrous) and sodium-2-ethyl hexanoate. The amount of cation source added and the reaction duration is dependent of the concentration of the (HMG)-CoA reductase inhibitor. Persons skilled in the art can select a proper species and amount of the cation source to obtain the salt form of the inhibitor. Preferably the cation source is added in an concentration of 0.2 to 5.0 M with stirring for 0.5 to 2.0 hours.

Without any intention to limit it in any manner, the present invention will be further illustrated by the following examples.

20

EXAMPLES

Example 1

A. Isolation of strains YS-44442 and YS-45494 from soil samples

Soil samples used for the isolation of actinomycetes were collected

from random sites. The soil samples were kept at room temperature until they dried to constant weight. One gram of an air-dried soil sample was suspended in 10ml of sterile 5mM phosphate buffer (pH 7.0) and stirred for 1 min in a super mixer. To germinate actinomycetes spores, the suspension of the soil sample was heated at 35°C to 50°C for 10 min in a shaking incubator. After a 10-fold serial dilution, the suspension was spread on agar plates containing suitable isolation medium. Colonies occurring on the plates were counted with naked eyes and examined under a light microscope for the morphological characteristics of actinomycetes. 400 strains showing positive morphological characteristics were selected from 3000 unidentified colonies. Due to a high conversion efficiency (>50%) from compactin to pravastatin and low epi-pravastatin content (<10%), strains *YS-44442* and *YS-45494* were selected among the 400 positive strains. The isolation conditions of *YS-44442* and *YS-45494* are described in Table 1.

Table 1: Isolation conditions of strains *YS-44442* and *YS-45494*

strain no.	<i>YS-44442</i>		<i>YS-45494</i>	
Soil source	Taipei, Taiwan		Australian desert	
Soil pH	5.4		9.6	
pretreatment	moist heat at 50°C for 10 min		moist heat at 40°C for 10 min	
Selective antibiotics	Nystatin	50μ g/ml	Nystatin	50μ g/ml
	Cycloheximide	50μ g/ml	Cycloheximide	50μ g/ml
	Pen G	1μ g/ml	Gentamycin	1μ g/ml
	Polymyxin B	5μ g/ml		

B. Characteristics of strains YS-44442 and YS-45494

Strain YS-44442 or YS-45494 was maintained on slants of yeast extract-malt extract agar (ISP-2) [Shirling, E.B. and Gottlieb, D., *International Journal of Systematic Bacteriology*, 16(3): 313-340 (1966)] and grew at 27°C. Inoculum for physiological tests and preparation of biomass for DNA extraction were prepared by growing in tryptone-yeast extract broth (ISP-1) [Shirling and Gottlieb *supra*].

(1) Morphology and pigment production

Morphology characterizations were performed as described by Shirling & Gottlieb *supra*. Culture morphology was examined by light microscopy using a Nikon Optiphot-2 microscope equipped with 45× and 100× super-long working distance objectives. Color determination of the aerial mycelium was made by observation of the mature, sporulating aerial surface growth. Color determination of the substrate mycelium and diffusible soluble pigments, other than melanins, was made by observation of the reverse side. Substrate mycelium coloration was assigned to one of the following color groups: (1) yellow-brown; (2) yellow-brown+red (or orange); (3) yellow-brown+blue (or violet); (3) yellow-brown+green. Pigment and color determinations were defined based on comparison to the Tektronix© RGB color sampler (Xerox, Inc.). Morphological characterizations were carried out on yeast extract-malt extract agar (ISP-2), oatmeal agar (ISP-3), inorganic salts-starch agar (ISP-4), and glycerol-asparagine agar (ISP-5) [Shirling and Gottlieb *supra*].

With respect to the results of YS-44442, on ISP-2 and ISP-4, short, flexuous (open) hooked or (short) spiraled chains of 5 to 10 spores were

observed. The spores were smooth and spherical and were appropriately 2 to 3 times the hyphal diameter. Pseudosporangia were present on ISP-2 and ISP-4 but were more abundant on ISP-4. Aerial mass coloration was white (R100 G100 B100) on ISP-2 and ISP-3. No aerial mycelia were produced on either ISP-4 or ISP-5 (see Table 2). In addition, the coloration of the substrate mycelium of *YS-44442* was brown (R85 G70 B40, color 2) on ISP-2, ISP-3 and ISP-4, and yellow-brown (R100 G100 B70, color 1) on ISP-5 (see Table 2). Pigments did not change in response to pH changes following addition of approximately 100 μ l of 0.05 N NaOH and 0.05 N HCl. Furthermore, no soluble pigments were produced by *YS-44442* (see Table 2).

As to the result of *YS-45494*, aerial mass coloration of the aerial mycelium was white (R100 G100 B100) on ISP 2, 3, 4, and 5. On ISP-2, the aerial mycelium was long, straight, and branched. On ISP-3, the aerial mycelium coalesced with some swelling evident at the tips of the hyphal vegetative growth. Upon aging, strain A5 fragmented into cocco-vassillary elements on ISP-5. The coloration of substrate mycelium was orange-brown (R100 G85 B40, group 2) on ISP-2, red-brown (R70 G55 B40, group 2) on ISP-3, light yellow-brown (R100 G100 B85, color group 1) on ISP-4, and light yellow-brown (R100 G100 B70, group 1) on ISP-5 (see Table 2). Pigments did not change in response to pH changes following addition of approximately 100 μ l of 0.05 N NaOH and 0.05 N HCl. In addition, soluble pigments were observed on ISP-3 and ISP-5. Coloration was brown (R70 G55 B40) on ISP-3 and yellow (R100 G100 B85) on ISP-5 (see Table 2). Neither soluble colors changed in response to pH changes following addition of approximately 100 μ l of 0.05 N NaOH

and 0.05 N HCl.

The morphological characteristics observed of *YS-44442* and *YS-45494* are summarized in Table 2.

Table 2: Colony morphology of strains *YS-44442* and *YS-45494*

<i>YS-44442</i>			
2	White	Brown (group 2)	None
3	White	Brown (group 2)	None
4	None	Brown (group 2)	None
5	None	yellow-brown (group 1)	None
<i>YS-45494</i>			
ISP Medium	Aerial mass color	Substrate mycelium color	Soluble colors
2	White	Orange-brown (group 2)	None
3	White	Red-brown (group 2)	Brown
4	White	Light yellow-brown (group 1)	None
5	White	Light yellow-brown (group 1)	Yellow

5 (2) Physiological tests

Melanin production and sole carbon source utilization were determined as described by Shirling & Gottlieb *supra*. Melanin production was evaluated on peptone-yeast extract iron agar (ISP-6) and tyrosine agar (ISP-7) [Shirling & Gottlieb *supra*]. Sole carbon source utilization was evaluated on basal mineral salts agar (ISP-9) amended with the following to 1 % (w/v) concentration: D-glucose (positive control),

i-arabinose, sucrose, D-xylose, i-inositol, D-mannitol, D-fructose, rhamnose, raffinose, and cellulose [Shirling & Gottlieb *supra*]. The negative control consisted of un-amended basal mineral salts agar. Results were scored as described by Shirling & Gottlieb [Shirling & Gottlieb *supra*].

The results of the general growth characteristics of *YS-44442* and *YS-45494* are given in Table 3.

Table 3: General growth characteristics of *YS-44442* and *YS-45494*

Strain	<i>YS-44442</i>	<i>YS-45494</i>
ISP medium	Growth	Growth
2	Moderate	Moderate
3	Moderate	Moderate
4	Fair	Fair
5	Fair	Fair
6	Fair	Fair
7	Fair	Fair

With respect to the carbohydrate utilization patterns of *YS-44442*, strongly positive utilization was observed on L-arabinose, sucrose, D-fructose, d-fructose and rhamnose. Doubtful utilization was observed on cellulose and negative utilization was observed on I-inositol and raffinose. No melanoid pigments were produced by the culture.

As to the carbohydrate utilization patterns of *YS-45494*, strongly positive utilization was observed on L-arabinose, sucrose, and D-fructose. Positive utilization was observed on rhamnose and raffinose. Doubtful

utilization was observed on D-xylose, I-inositol, D-mannitol, and cellulose. No melanoid pigments were produced by the culture.

The results of the carbohydrate utilization patterns of *YS-44442* and *YS-45494* are given in Table 4.

5 Table 4: Carbohydrate utilization patterns

Strain	<i>YS-44442</i>			<i>YS-45494</i>		
Day	10	13	16	10	13	16
none	-	-	-	-	-	-
D-glucose	++	++	++	++	++	++
T-arabinose	++	++	++	+	+	++
sucrose	++	++	++	++	++	++
D-xylose	++	++	++	-	+/-	+/-
i-inositol	-	-	-	+/-	+/-	+/-
D-mannitol	++	++	++	+/-	+/-	+/-
D-fructose	++	++	++	+	+	++
rhamnose	++	++	++	+	+	+
raffinose	-	-	-	+	+	+
cellulose	-	-	+/-	+/-	+/-	+/-

++ = strongly positive utilization

+ = positive utilization

+/- = utilization doubtful

- = utilization negative

(3) 16s rDNA sequence determination

DNA extraction

Biomass for DNA extraction was prepared by growing in 50 ml of tryptone-yeast extract broth (ISP-1) for 10 days, with continuous shaking at 250 rpm on an orbital shaker (1-inch throw), at 27°C [Shirling & Gottlieb *supra*]. The liquid culture was harvested by centrifugation for 15 minutes at 15,000 × g. The biomass was transferred to a sterile mortar and pestle, and gently ground into a paste. Approximately 0.4 ml of TE buffer (10mM tris: 1mM ethylenedinitrotetraacetic acid) was added and the buffer and cell debris were transferred to a sterile 2-ml centrifuge tube with 100 µl of 10% SDS (sodium dodecyl sulfate) solution. The mixture was incubated in a water bath at 65°C for 15 minutes. Approximately 0.5 ml of buffered phenol (pH 8) [Ausubel, F. M., *Short protocols in molecular biology*, 3rd ed (1995), New York, Wiley. I v. (various pagings)] was added to the tube and mixed for 10 minutes. The mixture was centrifuged at 14,474 × g for 15 minutes. The aqueous layer was transferred to a sterile 2-ml centrifuge tube and 0.5 ml of chloroform-isoamyl alcohol (24:1) was added. The mixture was centrifuged at 14,474 × g for 15 minutes and the aqueous layer was transferred to a sterile 2-ml centrifuge tube. Approximately 50 µl of 5 M NaCl and 1 ml of cold 95 % ethanol were added to the tube, and the DNA was precipitated by holding the mixture -20°C for 24 hours. The mixture was centrifuged at 14,474 × g, for 30 minutes at 4°C, and the supernatant removed. Approximately 100 µl of 80 % ethanol was added to the tube followed by centrifugation at 14,474 × g for 10 minutes. The ethanol was removed and the pellet was dried for 30 minutes and re-suspended in 50 µl of sterile deionized water (18 Mohm).

The DNA was stored at -20°C until ready for use.

16s rDNA sequence determination

PCR amplification of the 16s rDNA sequence was performed on the extracted DNA using GibcoBRL® native Taq polymerase on a Perkin-Elmer GeneAmp® PCR System 2400. Purification of the amplified 16s rDNA was accomplished with the Bio-Rad Prep-A-Gene® DNA Purification System. Fluorescent labeled DNA for sequencing was prepared using the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit. DNA sequence was done on an ABI Prism® 310 Genetic Analyzer equipped with a 61 cm × 50 µm capillary using the ABI Prism® POP-6™ Performance Optimized Polymer 6 according to manufacturer's instructions. Primers GMG 1 and GMG 10 were used in 16s rDNA PCR amplification and sequencing (see Table 5). The 16s rDNA PCR amplification program is as follows: (1) 94°C for 7 minutes; (2) 94°C for 1 minute; (3) 53°C for 1 minute; (4) 72°C for 2 minutes; (5) 72°C for 6 minutes; steps 2 to 5 were repeated for 30 cycles; and (6) Hold at 4°C.

Table 5: Primers used in 16s rDNA PCR amplification and sequence

Primer	Direction	Sequence (5'⇒3')
GMG 1	27 Forward	GAG TTT GAT CCT GGC TCA G (SEQ ID NO: 3)
GMG 2	1385 Reverse	CGG TGT GTR CAA GGC CC (SEQ ID NO: 4)
GMG 3	1114 Forward	GCA ACG AGC GCA ACC C (SEQ ID NO: 5)
GMG 4	907 Reverse	CCG TCA ATT CAT TTG AGT TT (SEQ ID NO: 6)

GMG 5	803	ATT AGA TAC CCT GGT AG (SEQ ID NO: 7)
	Forward	
GMG 6	536	CAG CMG CCG CGG TAA TWC (SEQ ID NO: 8)
	Forward	
GMG 7	519	Reverse GWA TTA CCG CGG CKG CTG (SEQ ID NO: 9)
GMG 8	357	TAC GGG AGG CAG CAG (SEQ ID NO: 10)
	Forward	
GMG 9	343	Reverse CTG CTG CCT CCC GTA (SEQ ID NO: 12)
GMG 10	1525	AGA AAG GAG GTG ATC CAG CC (SEQ ID NO: 13)
	Reverse	

5 The 16s rDNA sequences of *YS-44442* and *YS-45494* comprise the sequences as shown in SEQ ID NO: 1 and 2, respectively. The sequences were aligned against the multiple sequence alignment dataset in the Ribosomal Database Project (RDP) [Maidak, B.L. et al., *Nucleic Acids Res.*, 28: 173-174 (2000)] using the ARB sequence editor (release 8.1). Comparisons of the 16s rDNA sequences of *YS-44442* and *YS-45494* to the RDP [Maidak, B.L. et al., *supra*] shows 99.1 % and 99.0 % sequence similarity, respectively, to *Saccharothrix sp.* NRRL B-16133 [Labeda, D.P. and R.M. Kroppenstedt, *Int. J. Syst. Evol. Microbiol.*, 50 Pt 1: 331-6 (2000)] . (<http://www.eme.msu.edu/RDP/html/index.html>). In addition, 10 16s rDNA phylogenetic distance tree, comparing *YS-44442* and *YS-45494* with related actinomycetes (Table 6), generated with the ARB software package (denoted as tree 0.1) is shown in Figure 1.

Table 6: Actinomycetes related to strains *YS-44442* and *YS-45494*

GenBank Accession	Organism	Tree Designation
U48842	<i>Nonomuraea Africana</i>	Nm. african

AB006156	<i>Sebekia benihana</i> strain IFO14309	AB006156
U48977	<i>Nonomuraea polychroma</i>	Nm. plychrm
U48975	<i>Nonomuraea helvata</i>	Nm. helvata
U28978	<i>Nonomuraea pusilla</i>	Nm. pusill2
U48843	<i>Nonomuraea angiospora</i>	Nm. angiosp
X97892	<i>Nonomuraea salmonea</i>	Nm. salmone
AB006158	<i>Cathayosporangium alboflavum</i> strain IFO16009	AB006158
U48999	<i>Streptosporangium vulgare</i>	Sts. vulgar
X99942	<i>Streptomyces vellosus</i>	X99942
X60514	<i>Streptomyces coelicolor</i>	Stm. cocli3
X79853	<i>Streptomyces hygroscopicus</i>	Stm. hyscop
D85121	<i>Streptomyces virginiae</i> strain ATCC 13161	Stm. virgi5
Y15502	<i>Streptomyces griseus</i>	Y15502
X53194	<i>Saccharopolyspora rectivirgula</i>	Scp. rectiv
X53198	<i>Saccharopolyspora erythraea</i>	Scp. erythr
AF154128	<i>Saccharopolyspora flava</i>	AF154128
AF002818	<i>Saccharopolyspora spinosa</i>	Scp. spinos
AB020031	<i>Saccharothrix tangerinus</i> strain MK27-91F2	AB020031
AF114804	<i>Saccharothrix aerocolonigenes</i>	AF114804
AF114815	<i>Saccharothrix texasensis</i> strain NRRL B-16107T	AF114815
AF114807	<i>Saccharothrix espanaensis</i>	AF114807
X76966	<i>Saccharothrix mutabilis</i> ssp. <i>mutabilis</i> DSM 43853	Sct. mutabm

X76965	<i>Saccharothrix mutabilis</i> ssp. <i>capreolus</i> DSM 40225	Sct. mutabc
M29282	<i>Saccharothrix australiensis</i>	Sct. austra
AF114803	<i>Saccharothrix australiensis</i>	Sct. austr2
AF114812	<i>Saccharothrix syringae</i>	AF114812
AF114805	<i>Saccharothrix coeruleofusca</i>	AF114805

C. Comparison of YS-44442 and YS-45494 with known microorganisms

YS-44442 and YS-45494 both exhibit phenotypic characteristics similar to those described by Bergey's Manual of Systematic Bacteriology [Cross, T., *supra*] for *Saccharothrix aerocolonigenes* and *Saccharothrix australiensis*, and those described by Labeda and Lyons [Labeda, D.P. and Lyons, A.J., *International Journal of Systematic Bacteriology*, 39(3): 344-358 (19689)] for *Saccharothrix texasensis* (Table 7). YS-44442 and YS-45494 differ in morphology and carbohydrate utilization patterns from published descriptions (Table 8). They also differ in carbohydrate utilization patterns with *Saccharothrix aerocolonigenes*, *Saccharothrix australiensis*, and *Saccharothrix texasensis*, (Table 8).

Table 7: Comparison of *YS-44442* and *YS-45494* to the descriptions of related microorganisms described in Cross, T. *supra* and Labeda and Lyons *supra*

		<i>Saccharothrix aerocolonigenes</i>	<i>Saccharothrix australiensis</i>	<i>Saccharothrix texasensis</i>	<i>YS-44442</i>	<i>YS-45494</i>
Aerial Mycelium	Color	white	white to yellowish-gray	white	white	white
	Morphology	fragmented	fragmented into coccoid elements	no description	no description	fragmented into cocco-vassilary elements
Spore cham	Morphology	no description	no description	no description	short theyous hooked or spiral spore chains	no description
	Number	no description	no description	no description	5-10 spores cham	no description
Spores	Morphology*	no description	no description	no description	smooth and spherical and 2-3X the hyphal diameter	no description
Substrate Mycelium	Color	yellowish to brownish	brownish to yellowish-gray	dark yellow to brownish yellow	brown to yellow-brown depending on the growth medium	orange-brown, red-brown or light yellow-brown depending on the growth medium
	Morphology	branched	fragmented into coccoid elements	no description	presence of Pseudosporangia	some swelling at the tips of the hyphal vegetative growth

Soluble pigment	Color	yellowish to brownish	brownish	brown to reddish brown	none	brown or yellow depending on the growth media
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*: as viewed by light microscopy

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Table 8: Comparison of *YS-44442* and *YS-45494* to the carbohydrate utilization pattern of related microorganisms described in Cross, T. *supra* and Labeda and Lyons *supra*

Utilization of carbon compounds	<i>Saccharothrix aerocolonigenes</i>	<i>Saccharothrix australiensis</i>	<i>Saccharothrix texasensis</i>	Strain <i>YS-45494</i>	Strain <i>YS-44442</i>
no carbon control	-	-	-	-	-
D-glucose	+	+	+	+	+
L-arabinose	+	-	+	+	+
sucrose	+	-	+	+	+
D-xylose	+	-	+	-	+
I-inositol	-	-	+	-	-
D-mannitol	no information	no information	no information	-	+
D-fructose	+	+	variable	+	+
Rhamnose	-	-	+	+	+
Raffinose	-	-	-	+	-
Cellulose	-	-	no information	-	-

Based on the combined results of phylogenetic and physiological analyses, we believe strains *YS-44442* and *YS-45494* are distinct from one another and from the type strains of *Saccharothrix aerocolonigenes*, *Saccharothrix australiensis*, and *Saccharothrix texasensis* and are novel species of the genus *Saccharothrix*. The two strains were deposited with the China Center for Type Culture Collection (CCTCC) and given the accession number of M202001 and M202002, respectively, on 8 January 2002.

Example 2

Production of pravastatin by YS-44442 and YS-45494

(1) Preparation of compactin solution to be added in a fermentation broth

0.3 N NaOH solution (500 ml) was warmed and kept at 50 to 60°C.
5 The NaOH solution was added with 40g compactin and mixed for 2 to 3 hours at 50 to 60°C. The compactin solution in NaOH was cooled down to room temperature and the pH was adjusted to 7.5 with 1N HCl. The volume of the compactin solution in NaOH was adjusted to 1000 ml and centrifuged at 1000 rpm for 20 minutes. The supernatant was sterile by
10 filtration.

(2) Production of pravastatin using YS-44442 and YS-45494

1 ml of a spore suspension of YS-44442 (or YS-45494) stored in GL(10% glycerol , 5% lactose) at -80°C was opened in laminar flow and added into a 300 ml shake flask containing 20 ml of the seed medium (for
15 every liter: glucose 10 g, peptone 2 g, soy protein 4 g and KH₂PO₄ 1 g, pH 7.0±0.2). The seed culture was incubated at 27°C for 24 to 48 hrs in a rotary shaker at 200 to 220 rpm (growth phase). Afterward, 1.5 to 2 ml of the seed culture was incubated into a 300 ml shake flask containing 20 ml of the fermentation medium (per liter: glucose 15 g, peptone 5 g, corn steep
20 powder (C.S.P.) 5 g and soybean meal 4 g, KH₂PO₄ 1 g, pH 7.0±0.2). The shake flask was incubated at 27 ± 0.5°C for 18 to 24 hrs in a rotary shaker at 200 rpm (growth phase). The fermentation broth was added with compactin prepared as above into the fermentation broth at a final concentration of 1500 to 2000 mg/L and the incubation was continued for

16 to 24 hrs.

(3) HPLC analysis of pravastatin and compactin

The fermentation broth (0.2 ml) was added into 1.8 ml deionized water. The mixture was mixed for 1 minute and centrifuged at 3000 rpm for 10 minutes. The supernatant was analyzed by HPLC using the following conditions.

Column: Nuclosil₅ C₁₈, 5 μ m(4.6*150mm)
 Mobile phase: A:0.1 % (H₃PO₄/CH₃CN/CH₃OH=60/32/22)
 (*gradient) B:CH₃CN
 Inject volume: 10 μ l
 Flow rate: 1.0 ml/min
 Detection: 240 nm
 Retention time: Epi-pravastatin (pravastatin epimer) : 6.25 min
 pravastatin : 6.85 min
 compactin : 9.85 min

* The mobile phase was flow with the following gradient:

Time(min)	Flow	%A	%B
0	1.0	100	0
1.0	1.0	100	0
1.1	1.0	30	70
3.5	1.0	30	70
3.6	1.0	100	0
30	1.0	100	0
31	0.0	100	0

(4) Calculation and Results

A standard pravastatin or compactin preparation dissolved in alkaline methanol (100 % methanol and 0.1 N NaOH) and diluted with

H₂O were prepared. Pravastatin yield, molar conversion efficiency (ME %) and epi-pravastatin ratio (Epi %) are calculated by Formula (I), (II) and (III) as stated above.

5 The conversion efficiency of compactin to pravastatin, the pravastatin yield and the epi-pravastatin ratio of *YS-44442* and *YS-45494* are shown in Table 8.

Table 8: Results of Pravastatin production by strains *YS-44442* and *YS-45494*

Strain	Pravastatin yield	conversion efficiency	Epi-pravastatin ratio
<i>YS-44442</i>	1.0 to 1.2 g/L	70 to 75 %	6.5 to 8 %
<i>YS-45494</i>	1.0 to 1.5 g/L	48 to 50 %	1.8 to 3.0 %

Example 3

10 Production of pravastatin by *YS-44442* and *YS-45494* with continuous feeding of compactin

Compactin solution to be added in a fermentation broth was prepared as described in Example 1. A spore suspension of *YS-44442* or *YS-45494* (9 ml) stored in GL (10% glycerol + 5% lactose) at -80°C was
15 opened in laminar flow and added into a 3 L shake flask containing 600 ml of the seed medium as described in Example 1. The seed culture was incubated at 27°C for 24 to 48 hrs in a rotary shaker at 200 to 220 rpm (growth phase). Afterward, 1.6 L of the seed culture was incubated into a 30 L fermentor containing 16 L fermentation medium as described in
20 Example 1 in which 0.1 ml antifoam (LG-109) per liter was further added. The fermentation was conducted at 27°C with an agitation at 100 to 150

rpm and an air supply of 0.9 volume air per volume broth per minute for 24 to 48 hrs. Afterward, compactin (50 to 60 g/L) was fed into the fermentation broth in an amount of 10 to 30 ml/hr. After 2 to 3 days of feeding, the fermentation was finished. The fermentation broth was analysis by HPLC as described in Example 1, and the conversion efficiency of compactin to pravastatin, the pravastatin yield and the epi-pravastatin ratio of YS-44442 and YS-45494 are calculated as stated in Example 1 and shown in Table 9.

Table 9: Results of Pravastatin production by YS-44442 and YS-45494 with continuous feeding of compactin

Strain	Pravastatin yield	conversion efficiency	Epi-pravastatin ratio
YS-44442	1.0 to 1.5 g/L	70 to 75 %	6.5 to 8 %
YS-45494	1.0 to 1.5 g/L	48 to 50 %	1.8 to 3.0 %

In comparison with the known microorganisms (e.g., *Actinomadura sp.* as described in Peng, M. et al., J. Antibiotics, *supra* and Peng Y. and A. L. Demain, *supra*) which need a conversion period of 5 to 7 days (after compactin addition) to produce a yield of 0.326 to 0.821 g/L of pravastatin with a compactin feeding concentration of 0.5 to 1.1 g/L, YS-44442 and YS-45494 of the invention are capable of producing a yield of 1.0 to 1.5 g/L of pravastatin within 24 hours (after compactin addition) with a compactin feeding concentration of 1.5 to 2.0 g/L. In addition, compactin can soon be added into a fermentation broth of YS-44442 or YS-45494 after the fermentation broth is cultured for only 18 hours (from inoculation with a seed culture). In the prior art, compactin cannot be added into a fermentation broth of *Actinomadura sp.* (Peng, M. et al., J. Antibiotics,

supra and Peng Y. and A. L. Demain, *supra*) unless the broth is cultured for more than 2 days (from inoculation with a seed culture). Further, the pravastatin produced by YS-44442 or YS-45494 contains low ratio of epi-pravastatin (i.e., 6 α -hydroxylation form of compactin) which does not
5 have a desired therapeutic efficacy and even has a negative effect in clinical therapies. The two novel microorganisms of the invention exhibit a higher tolerance to compactin and a better conversion efficiency of compactin to pravastatin.

Example 4

10 NTG mutagenesis

A spore suspension of YS-44442 or YS-45494 was added into 10 ml of ISP2 medium and incubated with shaking for 1 to 2 hours. After centrifugation, the spore pellet was washed with 0.9 % NaCl twice and suspended in 3 ml of 0.9 % NaCl. 2.5 ml of 0.2 M NaOH and 1 ml of
15 1000 ppm NTG were added into the spore suspension, mixed well and incubated at $27 \pm 1^\circ\text{C}$ for 10, 20, 30, 60 and 120 min. After incubation, the spore suspension was added with 12% sodium thiosulfate and the up-layer after centrifugation was removed to eliminate the NTG. 10 ml of ISP2 medium was added into the spore suspension and incubated with
20 shaking for 1 to 2 hours. After centrifugation, the spore pellet was washed with 0.9% NaCl twice and suspended in 0.3 ml of 0.9% NaCl. After a 10-fold serial dilution, 0.1 ml of the diluted spore suspension was spread onto ISP2 agar plates. After incubating at $27 \pm 1^\circ\text{C}$ for 6 to 9 days, morphologies and the numbers of the colonies appearing on the plates were
25 observed and counted, respectively. Preferably, the killing rate

determined by the following formula was between 50 to 99.5%.

Killing rate = (numbers of cells without NTG treatment - numbers of cells through NTG treatment)/ numbers of cells without NTG treatment x 100%

5 For example, if the agar plate spread by 10^4 fold diluted spore suspension through NTG treatment appears 28 colonies (equivalent to 2.8×10^6 cells) and the agar plate spread by 10^6 fold diluted spore suspension without NTG treatment appears 36 colonies (equivalent to 3.6×10^8 cells), then the killing rate is about 99.22% [$100\% \times (360,000,000 - 2,800,000) / 360,000,000$]. Other mutagenesis using another mutagen can be conducted by the above method.

UV irradiation mutagenesis

15 A spore suspension of *YS-44442* or *YS-45494* was diluted by 10 fold serial dilution. 0.1 ml of diluted spore suspension was spread onto ISP2 agar plates. The agar plates were opened and treated with UV light for 30, 60, 120, 240 and 480 seconds. After UV irradiation, the agar plates were covered and immediately transferred into a black condition to avoid further light illumination. After incubating at $27 \pm 1^\circ\text{C}$ for 6 to 9 days, morphologies and numbers of the colonies appearing on the plates were observed and counted. Preferably, the killing rate determined by the following formula is between 99 to 99.999%.

Example 5

Bioactivity assay of mutants derived from *YS-44442* or *YS-45494*

After mutagenesis, the morphological variant was selected and put into screw tube with 6 to 10 glass beads and 0.5 ml deionized water, and mixed well. 0.1 to 0.15 ml of the suspension was refreshed in ISP2 slant agar and fermentated in a liquid medium. During fermentation, compactin was added in a final concentration of 1.5 or 2.1 g/L and the bioconversion was proceeded at 27°C for 24 to 48 hours. 0.2 ml of the fermentation broth was mixed with 1.8 ml deionized water. After centrifugation, the supernatant was analyzed by HPLC as described above. Mutants derived from *YS-44442* or *YS-45494* were selected for their pravastatin productivity, conversion efficiency and epi-pravastatin ratio (see Table 10).

Table 10: Bioassay results of mutants derived from strains *YS-44442* and *YS-45494*

Strain	Pravastatin yield	conversion efficiency	Epi-pravastatin ratio
44442(parent)	1.08 g/L	72.0 %	10.0 %
3600-1(mutant strain)	1.15 g/L	76.7%	8.2%
3600-2(mutant strain)	1.30 g/L	86.7%	5.0%
* Compactin is fed in a final concentration of 1.5 g/L			
45494(parent)	1.05 g/L	50.0 %	3.7 %
8400-1(mutant strain)	1.24 g/L	59.0%	3.0%
8400-2(mutant strain)	1.53 g/L	72.9%	2.1%
* Compactin is fed in a final concentration of 2.1 g/L			

Example 6

Purification of pravastatin by ammonium sulfate precipitation

A pravastatin-containing fermentation broth (14L, pH 7.5 to 8.5) of the mutant strain derived from the parent YS-44442 or YS-45494 was added with ammonium sulfate (4.5 to 7.0 kg) batchwise within 2 to 6 hours and then stirred for 5 to 15 hrs at room temperature to give a precipitation.

5 The precipitation thus produced was separated by filtration. The precipitation was dissolved in 100 to 200 ml of water and stirred for 0.5 hrs to produce a solution. The solution was filtered through a bed of celite, washing the celite bed once with 100 ml of water. The filtrate was collected and the pH of the filtrate was adjusted to pH 4.0 to 6.0 with 18 %
10 (w/v) HCl. The filtrate was then mixed with 1.0 to 3.0 volume of ethyl acetate and stirred for 10 to 30 min at room temperature. The emulsion was filtered by a bed of celite. After the phases were separated, the water phase was collected and extracted again with 1.0 to 3.0 volume of ethyl acetate. The ethyl acetate phases obtained from such twice extractions
15 were collected together. The collected ethyl acetate phases were dried and decolorized by adding with anhydrous magnesium (0.1 to 1.5 %w/v) and activated carbon (0.1 to 1.5 % w/v), stirring for 1 to 15 min at room temperature, filtering and washing with fresh ethyl acetate (100 ml), to yield 94.495 % (HPLC) of pravastatin.

20 To obtain a pravastatin sodium, the pravastatin purified as above was dissolved in fresh ethyl acetate to produce a solution containing pravastatin in a concentration of 5.0 to 25.0 g/L. The solution was added with 0.2 to 5.0 M sodium-2-ethyl hexanoate in methanol/ethanol or isopropyl alcohol and stirred for 0.5 to 2.0 hrs at room temperature. The solution was then
25 cooled to 0 to 20°C, filtered and washed with fresh ethyl acetate, yielding 60 to 80 % of pravastatin with a purity of 98.5 % (HPLC) wherein the

single impurity is in an amount of less than 0.3 % and the total impurity is less than 1.0 %.

2001/12/27